Clin Exp Immunol 1994; 96:26–30

The influence of anti-fibronectin antibodies on interactions involving extracellular matrix components and cells: a possible pathogenic mechanism

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(Accepted for publication 6 December 1993)

SUMMARY

Antibodies, directed to the 30-kD collagen binding domain (CBD) of fibronectin (Fn), have been previously demonstrated in sera from patients with systemic lupus erythematosus (SLE), and we now investigate the possible pathogenic effects of these antibodies on collagen–Fn and cell–Fn interactions. The binding of type 1 collagen to Fn was demonstrated by ELISA, and could be specifically inhibited by the preincubation of solid-phase immobilized Fn with anti-Fn antibodies from SLE sera. By using indirect immunofluorescent staining, anti-Fn antibody containing SLE sera but not normal human serum (NHS) reduced the deposition of newly synthesized collagen and Fn on living human skin fibroblasts. We also found that sera from SLE patients containing anti-Fn antibodies significantly reduced thyroid cell attachment to Fn immobilized on plastic compared with NHS. These effects were shown to be due to the presence of anti-Fn antibodies in these sera, as SLE sera depleted of anti-Fn antibodies did not reduce the deposition of collagen or Fn on cultured fibroblasts, nor did they inhibit cell attachment.

Keywords anti-fibronectin antibodies collagen binding domain extracellular matrix cell adhesion

INTRODUCTION

Fibronectin (Fn) is a multi-functional extracellular matrix (ECM) protein whose actions are mediated via the affinity of its domains for various macromolecules. At least five domains have been identified on Fn molecule, including one domain for fibrin-site 1, heparin-site 1 and Staphylococcus, and separate domains for collagen, cells, heparin-site 2, and fibrin-site 2 [1].

Anti-Fn antibodies have been demonstrated in a number of diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, a case of morphoea, and bacterial and certain viral infections [2–4]. In a previous study we have reported a prevalence of these antibodies, namely 28·7% of patients with SLE, and these antibodies were found to bind to a 30-kD fragment containing the collagen-binding domain of Fn (CBD) [3]. Only one CBD has been identified per Fn molecule and has been mapped to a region beginning about 30 kD from the amino-terminus of the Fn polypeptide [5,6]. Via this domain, Fn binds to all tested collagens, especially denatured type 1 collagen (gelatin), and it also binds to the collagenous portion of C1q and acetylcholine esterase [7–9]. The strength, specificity and selectivity of the collagen–Fn binding indicate that they could be physiologically significant, and it has been suggested that the binding of Fn to C1 has a role in the clearance of circulating immune complexes [10,11].

In this study we have investigated the influence of anti-Fn antibodies from SLE sera on the interactions between Fn and extracellular matrix components and cells.

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MATERIALS AND METHODS

Sera

Serum samples known to contain anti-Fn antibodies were obtained from four patients with SLE (patients 1–4). The human control sera were taken from an SLE patient lacking anti-Fn activity (patient 5) and a healthy individual (NHS). Goat anti-human Fn (Sigma) was used as the positive control (PC). Sera were stored at −70°C until use.

ELISA

Essentially we followed our previously described procedure for detecting anti-Fn activity in patients' sera [3]. Briefly, bovine Fn (Sigma) was coated on the wells of microtitre plates (Nunc) at 10 μg/ml carbonate/bicarbonate buffer pH 9·6 for 2 h at 37°C. The unbound Fn was removed with PBS containing 0·1% Tween 20 (PBS-T) and the residual binding sites were blocked with 2%

bovine serum albumin (BSA) in PBS for 1 h at room temperature. Uncoated wells were treated similarly with 2% BSA and used to detect non-specific binding. Sera diluted in PBS-T were then incubated in duplicate wells (coated and uncoated with Fn) for 2 h at room temperature. Alkaline phosphatase-conjugated goat anti-human immunoglobulins (recognizing α, μ and γ chains; Sigma) in PBS-T containing 1% BSA were incubated with all wells for 1 h at room temperature. After each step of the assay the wells were washed three times with PBS-T. Then the substrate solution (1 mg/ml *p*-nitrophenyl phosphate disodium (Sigma) in diethanolamine pH 9·8) was added and the optical densities (OD) were measured after 30 min at 405 nm by Multiskan ELISA reader. The result for each sample was expressed as an absolute OD and calculated using the following formula: Mean OD obtained on Fn-coated wells − mean OD obtained on uncoated wells.

The inhibition of antibodies binding to Fn immobilized on a microtitre plate, by type 1 collagen, was tested by adding calf serum type 1 collagen (Sigma) to the plate at 10 μg/ml in PBS before loading the patients' sera on the ELISA plate. Heparin 10 U/ml (CP pharmaceuticals) was added to duplicate wells as a control. The plate was then incubated for 2 h at 37°C, washed three times in PBS-T, and the assay continued as described above, starting with the addition of the patients' sera.

The blocking of collagen binding to Fn by anti-Fn antibodies was tested by incubating patients' sera on the plate before adding the calf serum type 1 collagen. Binding of type 1 collagen to Fn was then detected by the addition of rabbit IgG anti-human type 1 collagen (Chemicon) at 1:100 in PBS to all wells, and the plate was incubated for 2 h at room temperature. This was subsequently detected by a specific alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma). One hour later the wells were washed three times in PBS-T and the substrate solution was added to all wells. After 30 min incubation the OD was measured by the Multiskan ELISA reader.

Specific depletion of anti-Fn antibodies from patients' sera

Aliquots of sera diluted 1:10 in PBS-T were subjected to 10 consecutive incubations (1 h each) on microtitre wells precoated with the bovine Fn (by incubation with 10 μg/ml Fn for 2 h at 37°C and blocked with 2% BSA). Duplicate aliquots of sera were treated similarly on wells blocked with BSA but without Fn. The processed samples were tested for anti-Fn activity by ELISA, and were found to be reduced by between 64·6 and 82·6% of their original level, as judged by optical density.

Collagen and fibronectin deposition on cultured fibroblasts

Fibroblast culture. Human skin fibroblasts (BR 171; European Collections of Animal Cell Culture) were grown in 25-cm^2 tissue culture flask (Costar) at 37°C and 5% CO_2. The cells were maintained in 15% Fn-free fetal calf serum (FCS; Sigma) in complete RPMI medium, consisting of RPMI 1640 (Imperial Labs), 2 mmol L-glutamine, 10 mmol HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were supplemented daily with 25 μg/ml sodium ascorbate (Sigma). When the cells reached confluence, assessed by phase contrast microscopy, they were removed from the flask with 0·25% trypsin (Difco) in PBS. Aliquots of 500 μl of fibroblasts (2×10^4/ml) in complete RPMI medium with patients' or control sera (at final dilutions of 1:20) were added on to 13-mm diameter cover slips placed in wells of 24-well tissue culture plates (Costar). Duplicate wells were employed for each serum sample. After overnight incubation the production of type 1 collagen and Fn on fibroblasts on the cover slips was detected by indirect immunofluorescent staining.

Immunofluorescence staining for type 1 collagen. The cover slips were washed three times in RPMI 1640 medium, and the rabbit anti-human type 1 collagen (1:100 in PBS) was added to all cover slips (except the negative control, to which the complete RPMI was added). After 30 min humid incubation at room temperature, the cover slips were washed by rinsing once in 0·2% BSA in RPMI 1640 and twice in RPMI alone. Then FITC-conjugated goat anti-rabbit IgG (Sigma) was added to all cover slips. Following a further 30 min humid incubation the cover slips were washed three times as above and fixed with 5% acetic acid in ethanol for 5 min. Before mounting on glass slides, the cover slips were washed as before, but also with PBS. The deposition of collagen on fibroblasts was scored according to the intensity of the fluorescence.

Immunofluorescence staining for Fn. A similar procedure for collagen staining was followed apart from using goat anti-human Fn (1:100 in PBS) and rabbit anti-goat FITC conjugate (Sigma).

Cells adherence assay

Thyroid follicular cells. Human thyroid cells were grown to confluence in complete RPMI medium containing 10% Fn-free FCS and supplemented with 10 μg/ml insulin (Wellcome), 10 mU/ml bovine thyroid stimulating hormone, 10 ng/ml glycyl-l-histidyl-l-lysine acetate, 10 nM hydrocortisone and 5 μg/ml transferrin (Sigma). Thyroid cells were tested for cytokeratin, which is present in epithelial cells but not fibroblasts, by indirect immunofluorescence staining using anti-cytokeratin MoAbs (Sigma). More than 95% of the cells showed positive staining for cytokeratin. Thyroid cell suspension was made as described above for fibroblasts.

Cell adherence. The wells of 48-well tissue culture plates (Costar) were first coated with 10 μg/ml bovine Fn in PBS for 5 h at room temperature, then washed three times with PBS and blocked with 2% BSA for 18 h. A corresponding number of wells were blocked by BSA without prior coating with Fn. Serum samples were diluted 1:20 and incubated on wells coated with Fn and on uncoated wells for 2 h at room temperature. Aliquots of 5×10^4 thyroid cell suspension were then added to all wells and the plate was incubated for 45 min at 37°C. Next the plate was agitated for 30 s and the wells rinsed twice in PBS to remove unattached cells. The adherent cells were fixed with 0·3% paraformaldehyde in PBS and counted in three different random fields ($\times 250$ magnification) using a phase contrast inverted microscope. The results are presented as the mean $\pm$ s.d.

Statistical analysis

Student's *t*-test for paired data allowed comparison of the results of the cell attachment experiment.

RESULTS

Mutual inhibition of binding to fibronectin by collagen and anti-Fn antibodies

The binding of type 1 collagen to Fn coated on ELISA wells was detected by ELISA using specific anti-collagen antibodies

(Table 1). There was marked inhibition when SLE sera containing anti-Fn antibodies (patients 2 and 4) were incubated with the Fn before the addition of collagen; such inhibition was not observed with NHS (Table 1).

Conversely, the addition of collagen to the Fn before adding the patients' sera caused substantial reduction of antibody binding to Fn, while heparin failed to inhibit the binding of patients' anti-Fn antibodies to Fn (Table 2).

Inhibition of deposition of newly synthesized Fn and collagen on cultured fibroblasts by immunofluorescence staining

Sera containing anti-Fn antibodies caused a significant reduction in the deposition of Fn and collagen on fibroblasts after overnight incubation, compared with NHS (Table 3) as assessed by indirect immunofluorescence staining. Sera depleted of anti-Fn antibodies (as described in Materials and Methods and Table 1) did not reduce Fn or collagen deposition on fibroblasts (Table 3).

Table 1. Anti-fibronectin (Fn) antibodies inhibit collagen–Fn interaction in solid-phase ELISA*

Serum sample	OD	Per cent inhibition of† collagen binding by the sera
PBS (no serum)	0·370	Not applicable
NHS	0·280	24·3
Patient 4	0·080	78·4
Patient 2	0·160	56·8

* The sequence of addition onto Fn-coated ELISA plate was in the following order: sera (1:20) or PBS, type 1 collagen (10 μg/ml), rabbit anti-human type 1 collagen, anti-rabbit IgG enzyme conjugates.

† Per cent inhibition of collagen binding =

$$\frac{\text{mean OD with PBS} - \text{mean OD with human sera}}{\text{mean OD with PBS}} \times 100$$

NHS, Normal human serum; patient 4 and patient 2 sera are systemic lupus erythematosus (SLE) containing anti-FN antibodies.

Table 2. The binding of anti-fibronectin (Fn) antibodies in patients' sera is blocked by type 1 collagen but not by heparin*

Sera	OD without blocking	OD (% inhibition after blocking with) Collagen 1	Heparin
Patient 3	0·289	0·094 (67·4)	0·325 (0·0)
Patient 2	0·350	0·089 (74·5)	0·314 (10·3)
NHS	0·064	0·056 (12·5)	0·070 (0·0)

* Microtitre plates were coated with Fn and blocked with bovine serum albumin (BSA). Then type 1 collagen (10 μg/ml) or heparin (10 U/ml) was incubated in all wells for 2 h at room temperature. The wells were washed and sera (1:40) were added followed by the addition of the anti-human immunoglobulin conjugate followed by the substrate.

NHS, Normal human serum; patient 3 and patient 2 are systemic lupus erythematosus (SLE) sera containing anti-Fn antibodies.

Inhibition of cell adhesion to Fn-coated surface

Sera with anti-Fn activity caused a marked reduction in the number of the attached thyroid cells compared with NHS, and sera depleted of anti-Fn antibodies failed to inhibit cell adhesion. Table 4 demonstrates that non-depleted sera caused a significant reduction in the number of attached cells compared with depleted sera and normal human sera ($P < 0.005$).

Table 3. Inhibition of deposition of fibronectin (Fn) and collagen on cultured fibroblasts by anti-Fn-positive sera before and after depletion from anti-Fn activity

Sera	Collagen deposition* Depleted sera	Collagen deposition* Undepleted sera	Fn deposition* Depleted sera	Fn deposition* Undepleted sera‡
Patient 2	++	±†	++	±†
Patient 1	++	±†	++	+†
NHS	++	++	+++	+++
PC	ND	−†	ND	−†

* Fibroblasts (BR 171) were cultured overnight in the presence of the indicated sera (diluted 1:20), then stained for collagen or Fn deposition. Results expressed as per cent of Fn or collagen deposition on fibroblasts. −, No deposition noticed; ±, less than 25% of cells positive; +, 25–50% positive; ++, 50–80% positive; +++, more than 80% positive.

† Most cells failed to attach, and did not spread (rounded).

‡ Sera were depleted by incubation on Fn-coated wells, undepleted sera were treated in a similar way on bovine serum albumin (BSA)-coated wells.

NHS, normal human serum; PC, positive control (i.e. goat anti-human Fn); patient 2 and patient 1 are systemic lupus erythematosus (SLE) sera with anti-Fn antibodies.

Table 4. Inhibition of fibronectin (Fn)-mediated cell attachment by anti-Fn antibodies*

Sera	Mean no. of† attached cells (±s.d.) incubated with Anti-Fn depleted sera	Undepleted sera
Patient 2	161 (9·5)	44·3 (9·70)
Patient 1	192 (8·0)	46·0 (8·80)
Patient 5	133·6 (12·5)	134 (13·8)
NHS	121 (17·55)	110 (6·65)
PC	ND	4·6 (0·65)

* The wells of tissue culture plates were coated with Fn as described in Materials and Methods. Systemic lupus erythematosus (SLE) sera containing anti-Fn antibodies (patient 2 and patient 1), SLE serum lacking these antibodies (patient 5), normal human serum (NHS) and goat anti-human Fn-positive control (PC) were diluted 1:20 in PBS and incubated on the plate for 2 h at room temperature. The cell adherence study was performed as described in Materials and Methods. The results are expressed as the mean (±2 s.d.) of three different microscopic fields.

† Cell attachment is significantly inhibited by anti-Fn containing sera compared with NHS, SLE serum lacking the anti-Fn antibodies or sera depleted of anti-Fn antibodies ($P < 0.005$).

ND, Not done.

DISCUSSION

Previous studies demonstrated Fn–collagen (the main components of the ECM) *in vitro* interactions to occur only under critical conditions of temperature and be dependent on Fn's immobilization on a solid phase [12]. In this study, Fn–collagen binding has been demonstrated directly using anti-human type 1 collagen to detect collagen binding and indirectly by blocking of the binding of anti-Fn antibodies to Fn.

Conversely, SLE sera containing anti-Fn antibodies blocked type 1 collagen–Fn binding by 78·4% and 56·8% compared with 24·3% of NHS as measured by ELISA. This led us to study the effect of these antibodies on the deposition of the ECM proteins, Fn and collagen, on fibroblasts. We found a reduction in the deposition of freshly synthesized type 1 collagen and Fn on fibroblasts cultured with sera containing anti-Fn antibodies. We have also provided evidence that these effects were a consequence of the anti-Fn antibodies, as sera depleted of anti-Fn antibodies did not depress the deposition of collagen or Fn. These findings are consistent with our previous observations that patients' anti-Fn antibodies interact mainly to the CBD of Fn [3].

That anti-Fn antibodies can modulate Fn functions is of great interest when one considers that Fn is the backbone and the organizer for the deposition of ECM components. Fibronectin has binding sites for many of the ECM protein-like collagens, hyaluronic acid and heparan sulphate [1], and immunostaining studies have shown the occurrence of Fn along collagen fibrils. Removal of procollagens by enzymatic digestion from cultured fibroblasts does not interfere with Fn matrix [13] and the deposition of Fn on cultured fibroblasts has been shown to precede procollagens 1 and 3 [14–16]. Furthermore, treatment of living human skin fibroblasts with antibodies raised against the CBD disrupts the organization of both collagen and Fn [17].

The diminution of Fn deposition on fibroblasts by anti-Fn antibodies, that we noted, could be explained by inhibition of Fn–Fn interaction and cross-linking which is needed for elongation of Fn fibrils on cell surfaces. A Fn–Fn interaction site has been located near the CBD [18], whilst the cross-linking occurs by rearrangement of intramolecular bonds in the amino-terminal domain of the adjacent Fn molecules into intermolecular bonds [19]. Antibodies to the CBD of Fn may inhibit fibril formation by steric interference with the binding events involving the amino-terminal domain and the site of Fn–Fn interaction near the CBD. Indeed, binding of gelatin to the CBD was reported to inhibit the binding of macromolecules to the adjacent amino-terminus of the molecule [7]. From these *in vitro* studies, one might then hypothesize that the anti-Fn antibodies would lead to a reduction of collagen and Fn deposition. Both of these ECM proteins are known to be intimately involved in the structure and function of many organs, and their reduction in tissues could prove to be significant. Thus, functional weakness, reduced tensile strength and loss of tissue integrity may be a consequence of anti-Fn antibodies, and the anti-Fn antibodies may inhibit Fn from serving as a protein scaffold to direct tissue repair following inflammation. Although Fn is known to have an important role in wound healing, its role in parenchymal tissue injury is obscure [20]. Anti-Fn antibodies have been shown to cause *in vivo* pathology using laboratory animals. Rabbits and mice immunized with Fn preparations developed glomerular injuries [21,22], and rats injected with anti-Fn antibodies developed glomerular injuries [23]. Our preliminary results indicate an association between the occurrence of anti-Fn antibodies and active musculoskeletal involvement in SLE (manuscript in preparation).

We have also demonstrated that anti-Fn antibodies can inhibit thyroid cell attachment to Fn. This effect could not be related to blocking of Fn's cell-binding site(s) by the anti-Fn antibodies, because thyrocytes lack VLA-4 and express very low levels of VLA-5 [24], which are specific cell receptors required for binding with Fn's cell-binding sites. However, we have found that thyroid cells do express surface collagen (data not shown), and it is possible that these cells could bind through their surface collagen to Fn's CBD. Therefore the inhibition of thyroid cell attachment by anti-Fn antibodies could result from the direct blockade of the Fn's CBD.

The level of serum anti-Fn antibodies is an important indicator of their pathogenicity. However, serum levels of these antibodies might not reflect their local concentration in tissues, where it could be sufficient to cause tissue damage. It is possible that anti-Fn antibodies bind to the circulating Fn, which is elevated in SLE [25], forming undetectable (by our assay) immune complexes. However by using a specially designed ELISA we have detected these complexes in most anti-Fn-containing sera (data not shown).

In conclusion, we found that anti-Fn antibodies (by blocking the CBD) can inhibit collagen–Fn, Fn–Fn and cell–Fn interactions. These inhibitions could reduce tissue integrity and strength, and impact on the repair of tissue damaged by the inflammatory process. The effects of anti-Fn antibodies on the attachment, migration and proliferation of inflammatory cells deserve further investigation.

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